

Androgens and Environmental Antiandrogens Affect Reproductive Development and Play Behavior in the Sprague-Dawley Rat

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In mammals, exposure to androgens early in development is essential for masculinization of the male reproductive phenotype. Male fetuses exposed to antiandrogens during perinatal life are permanently demasculinized in their morphology and physiology, whereas exposure to exogenous androgens permanently masculinizes females. In some litter-bearing species, proximity *in utero* of females to males can partially masculinize female siblings and alter their responsiveness to endocrine-disrupting compounds. However, in our strain of rat (CD-SD Charles River), intrauterine position does not significantly influence testosterone concentrations and anogenital distance of fetuses. In comparison, administration of testosterone propionate to pregnant females, at doses that doubled fetal female testosterone levels, did masculinize the reproductive system. Discovery of androgen-active chemicals in the environment has placed increased emphasis on describing the reproductive and behavioral effects of both natural and environmental androgens and antiandrogens. Recently, the effects of an antiandrogen, vinclozolin, on the brain and behavior were cited as a special concern by the U.S. Environmental Protection Agency in its risk assessment of this pesticide. In rats, one such behavior that is perinatally organized by androgens is social play. Males play more than females, and administration of exogenous androgens during the neonatal period alters the juvenile expression of this sexually dimorphic behavior. Vinclozolin is an androgen receptor antagonist that inhibits androgen-dependent tissue growth *in vivo*. We were interested in whether developmental exposure to vinclozolin could also alter androgen-dependent behaviors such as play. Neonatal male rats were injected on postnatal days (PNDs) 2 and 3 with corn oil, the pharmacologic antiandrogen flutamide (50 mg/kg), or vinclozolin (200 mg/kg). On PNDs 36–37 animals were observed for social play. Behaviors associated with general social activity such as sniffing and dorsal contact were unaffected by treatment. However, play behavior in males treated with flutamide or vinclozolin was significantly reduced, resembling levels of play characteristic of females rather than untreated males. Therefore, this study demonstrates that perinatal exposure to vinclozolin, an environmental antiandrogen, can alter androgen-dependent play behavior in the male rat. **Key words:** androgens, anogenital distance, antiandrogens, endocrine-disrupting chemicals, intrauterine position, play behavior, reproductive development. *Environ Health Perspect* 110(suppl 3):435–439 (2002).

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Endocrine-Disrupting Chemicals

Since the early 1990s, significant attention has been focused on the potential for chemicals found in the environment to alter endocrine systems in animals including humans. This field of endocrine disruptors has quickly grown and now encompasses diverse areas of research such as immunology, toxicology, reproductive physiology, behavior, and ecology. Endocrine-active chemicals arise from many different sources, including pesticides, industrial chemicals, pharmaceuticals, and phytochemicals (Gray et al. 2001b). Currently, several well-documented examples of potent antiandrogens found in pesticides and plasticizers have been described (Gray et al. 2001b, 1994; Mylchreest et al. 1999; Parks et al. 2000). In addition, there are reports of masculinized fish in rivers in the United States and Europe resulting from potential androgen mimics in the environment (Parks et al. 2001).

In earlier work on environmental antiandrogens, investigators examined the role of chemicals that antagonized the function of androgen receptors (ARs). Prominent examples of AR antagonists are the pharmacologic agent flutamide as well as the fungicide vinclozolin (Gray et al. 1994, 1999; Imperato-McGinley et al. 1992). Both flutamide and vinclozolin act by binding to the AR and altering subsequent gene expression (Kelce et

al. 1997; Waller et al. 1996). Investigation of the effects of antiandrogens in developmental studies of male rats is consistent with AR antagonism. These effects include reduced anogenital distance (AGD), retention of nipples, genital malformations including cleft phallus and hypospadias, and sex accessory glands that were either reduced in mass or absent (Gray et al. 1994; Wolf et al. 2000). Furthermore, the critical window for inducing these changes appears to be around the same period for reproductive differentiation in fetal rats, namely gestational days (GDs) 16–19 (Wolf et al. 2000).

It is now apparent that a number of different mechanisms exist by which chemicals can alter reproductive development in mammals. For example, there is growing concern regarding the phthalate esters (PEs), now recognized as novel antiandrogens. Studies demonstrate that instead of blocking the androgen signal at the level of the AR, PEs reduce the production of testosterone by the

fetal testis (Mylchreest et al. 1998; Parks et al. 2000). Thus, PEs functionally act as antiandrogens during the prenatal period. Developmental effects of PEs include reductions in androgen-dependent tissues such as seminal vesicles, epididymis, prostate, and AGD—effects similar to those caused by known antiandrogens such as flutamide, procymidone, and vinclozolin (Gray et al. 1999; Mylchreest et al. 1998).

Finally, several examples of environmental androgens have been reported. In the Fenholloway River in Florida, mosquitofish exhibit masculinized gonopodia, with increased numbers of segments in the anal fin ray (a masculine trait) (Parks et al. 2001). Water samples from the same river are androgenic in *in vitro* assays. Parks et al. (2001) have shown that organic-soluble fractions of the water are able to bind and activate the human AR. These water samples were not able to activate a glucocorticoid-responsive reporter gene, implying independence

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from glucocorticoid receptor mediation. Additionally, reports have suggested a growth promoter used in livestock, trenbolone acetate, is a potent androgen that remains biologically active after discharge from the animal (Gray et al. 2001a; Schiffer et al. 2001).

As early exposure to exogenous steroids or their antagonists can have lasting effects on the adult physiology and behavior, prenatal sources of variation in androgen exposure must be identified. In addition, a behavioral assay for potential effects due to an early exposure to androgen-active chemicals should be developed. The following sections address these issues for the study of environmental androgens and antiandrogens in the rat.

Role of Androgens in Reproductive and Behavioral Development

In the rat, differentiation of the male reproductive phenotype depends on both Müllerian-inhibiting substance (MIS) and testosterone. Lack of expression of these two hormones results in the development of the female phenotype (Jost 1953). Whereas MIS is critical for the inhibition of the Müllerian system, testosterone is important for differentiation of the Wolffian ducts, associated sex accessory tissues, and testicular descent (Jost 1970). Testosterone, reduced by the enzyme 5 α reductase to dihydrotestosterone, is important for development of external genitalia and the prostate (Wilson and Lasnitzki 1971). Inappropriate expression or suppression of either MIS or testosterone results in abnormalities of the adult reproductive phenotype.

Behaviorally, hormones act prenatally to organize neural systems that later can be activated by the same steroid hormones and elicit behavior (Phoenix et al. 1959). Individuals are particularly sensitive to organizational effects of certain hormones during critical developmental periods. Whereas perinatal exposure to androgens is essential for masculinization of males, numerous studies have shown that females exposed to androgens during the appropriate perinatal period can be masculinized in morphology, physiology, and behavior (Huffman and Hendricks 1981; Rhees et al. 1997; Thornton and Goy 1986; vom Saal 1979).

In some species of mammals, the conversion of testosterone into estradiol by aromatase in the brain is critical for the organization of the male phenotype. For example, in the rat many of the organizing effects of testosterone also can be induced by administration of estradiol (Paup et al. 1972). This is possible because specific regions of the brain contain aromatase, which enables the conversion of testosterone into estradiol (Paup et al. 1972; Reddy et al. 1974). Inhibition of aromatase activity by

pharmacologic antagonists feminizes sexual behavior of male rats (Gladue and Clemens 1980; McEwen 1981). In addition, blocking the androgen pathway with antiandrogens does not affect organization of male-typical mounting behavior (Gray et al. 1994; Gray and Ostby 1998). Together, these studies suggest that the brain of the male rat is masculinized primarily by estradiol from the aromatization of testosterone. Although estradiol is critical for masculinization of some behaviors in the perinatal rat, others are affected specifically by androgens during the neonatal period. Play is organized by the perinatal interaction of androgens with the AR (Meaney et al. 1983; Meaney and McEwen 1986; Pellis and Pellis 1997). Although limited examples of androgen organization of behavior exist in the rat, it appears to be more common in the nonhuman primate (Cooke et al. 1998; Hines 1992). Developmental exposure to androgens in nonhuman primates enhances both male-typical expression of mounting behavior in adults as well as masculinization of sexually dimorphic regions of the brain (Cooke et al. 1998; Pomerantz et al. 1985; Thornton and Goy 1986).

Natural Variation in Androgen Concentrations during Development

Recognizing the importance of androgens for masculinization of physiology and behavior, investigators must identify sources of natural variability in exposure to perinatal androgens for males and females. Quantitative examination of androgens in fetal rats shows that males and females have similar androgen content during much of gestation (Baum et al. 1991; Weisz and Ward 1980). However, concentrations of androgens in males increase significantly late in gestation (Baum et al. 1991; Houtsmuller et al. 1995; vom Saal and Bronson 1980; Weisz and Ward 1980), reaching a peak around the critical developmental window of GDs 18–19 (Baum et al. 1991). Several studies have suggested that this brief exposure to elevated androgens late in gestation sensitizes the developing male to androgen exposure during the neonatal period (Baum et al. 1990; Hoepfner and Ward 1988; Tobet and Baum 1987). Therefore, potential variation in prenatal testosterone may interact with or alter the sensitivity to endocrine-disrupting chemicals (EDCs). However, even during this critical window, developing female fetuses have significant testosterone concentrations in many different tissues, including those critical for the developing reproductive system (Baum et al. 1991; Houtsmuller et al. 1995; Weisz and Ward 1980). In several cases, individual female concentrations of testosterone are

within the range experienced by males (Hotchkiss et al. 2000; Weisz and Ward 1980). Because prenatal administration of androgens masculinizes females and AR expression is comparable in males and females, it is unclear why females are not more masculinized (George and Wilson 1994). The source(s) of these androgens in female rats is not known, as there is no evidence that developing ovaries could be responsible for androgen production (Vreeburg et al. 1983). Several different extra-ovarian sources for the androgens in females have been proposed, including placenta (Baum et al. 1991; Vreeburg et al. 1983), fetal adrenals (Stahl et al. 1991), maternal circulation, or contribution by developing male fetuses (Clark et al. 1993; Drickamer et al. 1997; Vandenberg and Huggett 1995; vom Saal et al. 1999).

In several other litter-bearing species, variation in prenatal androgens can be largely attributed to the intrauterine position (IUP) phenomenon. The IUP refers to the random positioning of fetuses *in utero* such that a fetus can develop between two females (0M), a male and a female (1M), or between two males (2M). In these two species, the sex of the neighboring fetuses affects exposure to androgens and estradiol of the developing fetus (Clark et al. 1991; vom Saal and Bronson 1980). Females that develop between two males (2M females) are exposed to higher levels of androgens than females that develop between two females (0M females). In the rat, two different models, the contiguous and caudal model, for testosterone transfer from males to females have been proposed (Clemens et al. 1978; Even et al. 1992; Meisel and Ward 1981). These models arose from uncertainty as to the mechanism of androgen transfer from the fetal male to the fetal female. The contiguous model, similar to that in the mouse, states that testosterone from developing male fetuses diffuses through the fetal membrane to the neighboring animals. In comparison, the caudal male model states that maternal blood flows in the uterus in the rostral direction such that fetuses located caudally to males are exposed to increased levels of androgens.

In accordance with effects of prenatal testosterone, 2M female rodents show masculinized sexually dimorphic structures including the AGD and the sexually dimorphic preoptic area of the brain (Clark et al. 1993; Vandenberg and Huggett 1995; vom Saal et al. 1999). The AGD refers to the distance between the genital papilla and the anus; this region of skin is organized by androgens in the prenatal period, and as such, males have an AGD approximately twice that of females (Clemens et al. 1978; Gray et al. 1999; Vandenberg and Huggett

1995). Recently the AGD in the female mouse has been found to vary due to IUP (Vandenbergh and Huggett 1995). Similar correlations have been found in gerbils (Clark and Galef 1988) and swine (Drickamer et al. 1997).

The physiology and behavior of 2M females also differ from those of their less-masculinized female siblings. 2M females exhibit a delay in the onset of puberty, increased incidence of ovarian cycle irregularities, and accelerated onset of reproductive senescence (Clark and Galef 1998; Vandenbergh and Hotchkiss 2001; vom Saal et al. 1999). Behaviorally, masculinized female mice are more aggressive to intruders (vom Saal et al. 1999), are less attractive to males (vom Saal et al. 1999), and have a greater home-range size in the wild (Zielinski and Vandenbergh 1991). Interestingly, in both gerbils and mice, the intrauterine position of females has significant influence on their subsequent litter sex ratios. Females that develop *in utero* between two males produce more males in their litters than do females gestated between two females (Clark and Galef 1995; Vandenbergh and Huggett 1994). The mechanism for this alteration is uncertain but is suspected to occur either at the level of fertilization or implantation, as there is no evidence of selective postimplantation or postnatal loss (Clark and Galef 1995; Vandenbergh and Huggett 1994).

Recently, the IUP has been the focus of discussions in the toxicology community because of its potential to alter susceptibility of fetuses to endogenous hormones and EDCs (Clark et al. 1993; Howdeshell et al. 1999). In this regard, failure to account for IUP in EDC toxicology studies could lead to false negative results, especially when adverse alterations are produced by low doses in fetuses from only one IUP (Howdeshell et al. 1999; Welshons et al. 1999). This possibility has been raised because of investigations of estrogenic compounds in the mouse. In the rat, consistent effects due to IUP on testosterone concentrations, and therefore potential interactions with EDCs, have not been found. Contiguous (Clemens et al. 1978; Tobert et al. 1982), caudal (Houtsmuller et al. 1995; Richmond and Sachs 1984), and no effects (Baum et al. 1991; Hotchkiss et al. 2000; Meisel and Ward 1981; Slob and Van der Schoot 1982) due to IUP have been reported. Hotchkiss et al. (2000) in a recent study with Sprague-Dawley rats examined the effect of IUP on concentrations of testosterone in several different tissues. No effect of either contiguous or caudal IUP on testosterone concentration was detected in fetal carcass, reproductive tract, or amniotic fluid. Furthermore, no

correlation was found between masculinization due to IUP and increasing AGD. It is unclear at this time why there is such a discrepancy in results seen in the rat. However, varied strains of rat, multiple uncontrolled variables, and different criteria for defining the effects of intrauterine positioning may all contribute to this uncertainty. In addition, in one study the amount of variation in prenatal testosterone, which was explained by the IUP, was estimated to be less than 12% (Houtsmuller et al. 1995). Thus, differences in the rat may be relatively subtle and easily overshadowed by other more potent effects. Further, potential IUP effects on different hormones, their EDC counterparts, or at different developmental periods remain to be examined. Finally, the physiologic mechanisms underlying interspecies variation in IUP effects could be interesting and important subjects for future investigation.

Effect of Androgen-Active Chemicals on Behavior

As perinatal exposure to androgens has lasting effects on the morphology and physiology of the adult animal, investigators need to identify behaviors that could be used to examine behavioral effects of early developmental exposure to androgen-active EDCs. In rodents, many behaviors are masculinized by the conversion of testosterone into estradiol in the brain. However, some behaviors, such as rough-and-tumble play, are specifically organized by androgens. The critical time in the rat for organization of play is the early neonatal period, when interactions of androgens with the AR set up the sex difference observed in juveniles (Meaney et al. 1983; Meaney and McEwen 1986; Pellis and Pellis 1997). Studies have shown that increased male rough-and-tumble play is specifically organized by the stimulation of ARs in the amygdala region of the brain (Meaney et al. 1983) and is not dependent on activational effects of steroids (Meaney and McEwen 1986). Further, androgen receptor dysfunctions such as testicular feminized mutant males, neonatal castration, and neonatal exposure to the androgen-receptor antagonist flutamide decrease the expression of play by juvenile males, whereas administration of estradiol agonists does not increase play in either male or female rats (Meaney and Stewart 1981). Therefore, rat rough-and-tumble play is unique in that it is clearly organized by androgens themselves during the neonatal period and is not influenced by activational effects of other steroid hormones.

Although Meaney and Stewart (1981) showed that neonatal exposure to testosterone increased play in juvenile female rats, few studies have used play as a behavioral assay

for androgen-active environmental chemicals. This may be because of the widely held conception that most behaviors in the rat are organized by estradiol rather than androgens. However, research on the central nervous system (CNS) and behavior in primates indicates that androgens may play a larger role in differentiation in these species. Therefore, play behavior provides a straightforward rodent model for the effects of environmental chemicals on androgen-organized development.

For example, in a recent study (Hotchkiss et al. 2001), female neonatal rat pups were treated with either corn oil or 250 µg/kg/day testosterone propionate on PNDs 2 and 3; males were treated with corn oil, 50 mg/kg/day of the pharmacologic antiandrogen flutamide, or 200 mg/kg/day of the antiandrogenic fungicide vinclozolin. Play behavior was then observed for pairs of juvenile animals of the same litter, sex, and treatment. Animals were monitored for a period of 10 min, during which frequencies of social behaviors such as sniffing and rough and tumble play were noted. A play bout was defined as one in which an individual was pinned to the floor with another individual on top. Overall, a significant sex difference in play behavior between male and female juvenile rats was observed (Hotchkiss et al. 2001). Females treated with testosterone propionate had increased levels of rough-and-tumble play without alteration in the frequency of other social activities. In males, the AR antagonist flutamide significantly decreased the number of play bouts observed. Exposure to the fungicide vinclozolin also decreased play behavior in an antiandrogenic manner. Together, results of the androgen and androgen-receptor antagonist experiments support rough-and-tumble play as a neonatally androgen-organized behavior.

Several other reports have recently investigated the potential effect of antiandrogens on behavioral development in the rat (Flynn et al. 2001; Gray and Ostby 1998). In both of these studies, no effect of perinatal vinclozolin was found on male play behavior. However, differences in protocol between these studies could explain inconsistencies in results. First, in studies conducted by Flynn et al. (2001) and Gray and Ostby (1998), neonatal exposure to the AR antagonist was through lactational exposure. In the Hotchkiss et al. study (2001), the compound was administered directly to the pup. Second, the dose used in the Flynn study was lower (up to 60 mg/kg/day to pregnant female) than that used in the Hotchkiss et al. (200 mg/kg/day to neonatal pup) and Gray and Ostby studies (200 mg/kg/day to pregnant female). Finally, the Flynn et al. study observed play behavior in the first 6 hr of

lights on. During this period they did not see a significant sex difference in play. This is in contrast to the two other studies that observed higher levels of play behaviors in males than in females during lights out when activity levels are higher in nocturnal animals such as the rat. As behavioral testing of EDCs continues to expand, subtle differences in protocol must be considered and minimized to reduce seemingly contradictory outcomes.

Finally, concerns have been raised regarding the efficacy of using a neonatal rodent model requiring subcutaneous injections, as this route of exposure has limited applicability to primates. However, it is important to consider differences in timing of brain development in rats versus primates. Much of sexual differentiation of the brain in rats occurs during the first several days postpartum (Tobet et al. 1982). By comparison, in primates much of this differentiation occurs *in utero* (Cooke et al. 1998). Therefore, toxicants present in pregnant females potentially could be transferred to their offspring. Further, neonatal rat administration of vinclozolin likely would be easily absorbed because of the immature status of the gastrointestinal tract at this age (Klein 1986). Hence, subcutaneous and oral routes of administration are likely to provide similar internal exposures. However, because of species differences in the rates of CNS development in relation to the timing of birth, none of the standard routes of exposure (lactational, gavage, subcutaneous injection) simulates precisely what might occur in humans.

It is evident that studies are needed to determine concentrations of active metabolites of vinclozolin in the rat CNS during neonatal life. These concentrations then can be compared with estimated or measured (in nonhuman primates) concentrations resulting from transplacental exposures in long-term gestation animals. If concentrations of active metabolites are comparable between these species, then injections of endocrine-active chemicals into neonatal rats could constitute a meaningful route of exposure at the appropriate developmental period. Recent studies have emphasized the importance of the maternal/fetal transfer of toxicants. For example, Blount et al. (2000) showed that a cohort of women between 20 and 40 years of age had measurable amounts of endocrine-active chemicals in their urine. The question now becomes what, if any, adverse effects on the developing offspring result from these compounds?

As the EDC field continues to grow, other examples of chemicals interfering with masculinization of the brain could arise. This study has implications both for risk assessment of known antiandrogens,

such as vinclozolin, and for testing of novel chemicals. Although antiandrogenicity of vinclozolin has been established since 1994, a recent report by the U.S. Environmental Protection Agency (U.S. EPA) calls for studies of the developmental neurotoxicity of vinclozolin (U.S. EPA 2000). This lag in behavioral data on vinclozolin likely results from the limited numbers of behaviors masculinized by androgens, rather than estradiol, in the rodent. In addition, recent reports (Gray et al. 2001b; Parks et al. 2001; Schiffer et al. 2001) of androgenic chemicals in the environment necessitate development of behavioral tests for assessing organizational effects of these chemicals on females. Considering the utility of this assay for testing behavioral effects of these EDCs, play behavior could be developed into a rodent behavioral test for chemicals with androgenic or antiandrogenic activity.

Summary

Discovery of novel antiandrogens (Mylchreest et al. 1998; Parks et al. 2000) and reports of androgenic chemicals (Gray et al. 2001a; Parks et al. 2001; Schiffer et al. 2001) in the environment has renewed interest in describing the role of androgens in reproductive and behavioral development. Toward this end, this article has described initial studies addressing several important issues in this field. First, variation in the concentrations of testosterone in the developing fetus was discussed. Although IUP has been shown to explain much of the variability in prenatal androgens in the mouse and gerbil, results of current studies of IUP effects in the Sprague-Dawley rat suggest that it is not a significant source of variability in terms of testosterone and masculinization. Regardless of the sources of variation in prenatal androgens, several useful biomarkers have been suggested for characterizing the prenatal androgen environment (Gray et al. 1999; Vandenberg and Huggert 1995). These biomarkers include the androgen-sensitive AGD and areola/nipple formation. Both of these indicators reflect the prenatal androgen environment and can be used for characterizing early developmental exposure to androgenic or antiandrogenic chemicals.

Finally, social play is an androgen-organized behavior in rats that can be used to test for environmental androgens and antiandrogens. Assessment of developmental neurotoxicity is important for risk assessment of EDCs, as evidenced by the U.S. EPA call for more of these studies on vinclozolin. Play behavior provides a straightforward rodent model for assessing the effects of environmental chemicals on AR-organized development.

REFERENCES

- Baum MJ, Erskine MS, Kornberg E, Weaver CE. 1990. Prenatal and neonatal testosterone exposure interact to affect differentiation of sexual behavior and partner preference in female ferrets. *Behav Neurosci* 104:183–198.
- Baum MJ, Woutersen PJ, Slob AK. 1991. Sex difference in whole-body androgen content in rats on fetal days 18 and 19 without evidence that androgen passes from males to females. *Biol Reprod* 44:747–751.
- Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, et al. 2000. Levels of seven urinary phthalate metabolites in a human reference population. *Environ Health Perspect* 108:979–982.
- Clark MM, Crews D, Galef BG. 1991. Concentrations of sex steroid hormones in pregnant and fetal Mongolian gerbils. *Physiol Behav* 49:239–243.
- Clark MM, Galef BG. 1988. Effects of uterine position on rate of sexual development in female Mongolian gerbils. *Physiol Behav* 42:15–18.
- Clark MM, Galef BG Jr. 1998. Effects of intrauterine position on the behavior and genital morphology of litter-bearing rodents. *Dev Neuropsychol* 14:197–211.
- Clark MM, Galef BG. 1995. A gerbil dam's fetal intrauterine position affects the sex ratios of litters she gestates. *Physiol Behav* 57:297–299.
- Clark MM, Karpiuk P, Galef BG. 1993. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. *Nature* 364:712.
- Clemens LG, Gladue BA, Coniglio LP. 1978. Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. *Horm Behav* 10:40–53.
- Cooke B, Hegstrom CD, Villeneuve LS, Breedlove SM. 1998. Sexual differentiation of the vertebrate brain: principles and mechanisms. *Front Neuroendocrinol* 19:323–362.
- Drickamer LC, Arthur RD, Rosenthal TL. 1997. Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. *J Anim Sci* 75:2192–2196.
- Even MD, Dhar MG, vom Saal FS. 1992. Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. *J Reprod Fertil* 96:709–716.
- Flynn KM, Delclos KB, Newbold RR, Ferguson SA. 2001. Behavioral responses of rats exposed to long-term dietary vinclozolin. *J Agric Food Chem* 49:1658–1665.
- George FW, Wilson JD. 1994. Sex determination and differentiation. In: *Physiology of Reproduction* (Knobil E, Neill J, eds). New York: Raven Press, 3–28.
- Gladue BA, Clemens LG. 1980. Masculinization diminished by disruption of prenatal estrogen biosynthesis in male rats. *Physiol Behav* 25: 589–593.
- Gray LE Jr, Lambright C, Wilson V, Ostby J, Guillette LJ Jr, Wilson E. 2001a. *In vivo* and *in vitro* androgenic effects of beta Trenbolone, a potential feed-lot effluent contaminant. SETAC 22nd Annual Meeting, 15 November 2001, Baltimore, Maryland. Abstract. PH070, 334.
- Gray LE Jr, Ostby J. 1998. Effects of pesticides and toxic substances on behavioral and morphological reproductive development: endocrine versus nonendocrine mechanisms. *Toxicol Ind Health* 14:159–184.
- Gray LE Jr, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, et al. 2001b. Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update* 7:248–264.
- Gray LE Jr, Ostby JS, Kelce WR. 1994. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol* 129:46–52.
- Gray LE Jr, Ostby J, Monosson E, Kelce WR. 1999. Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol Ind Health* 15:48–64.
- Hines M. 1992. Surrounded by estrogens? Considerations for neurobehavioral development in human beings. In: *Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection* (Colborn T, Clement C, eds). Advances in Modern Environmental Toxicology, Vol XXI. Princeton, NJ: Princeton Scientific Publications, 231–259.
- Hoepfner BA, Ward IL. 1988. Prenatal and neonatal androgen

- exposure interact to affect sexual differentiation in female rats. *Behav Neurosci* 102:61–65.
- Hotchkiss, AK, Ostby J, Vandenberg JG, Gray LE Jr. Unpublished data.
- Hotchkiss A, Parks LG, Ostby J, Lambright C, Wolf C, Wilson VS, et al. 2000. A quantitative determination of the environmental sources of variability in androgens of fetal Sprague-Dawley rats [Abstract]. *Biol Reprod* 62:183.
- Houtsmuller EJ, de Jong FH, Rowland DL, Slob AK. 1995. Plasma testosterone in fetal rats and their mothers on day 19 of gestation. *Physiol Behav* 57:495–499.
- Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenberg JG, vom Saal FS. 1999. Exposure to bisphenol A advances puberty. *Nature* 401:763–764.
- Huffman L, Hendricks SE. 1981. Prenatally injected testosterone propionate and sexual behavior of female rats. *Physiol Behav* 26:773–778.
- Imperato-McGinley J, Sanchez RS, Spencer JR, Yee B, Vaughan ED. 1992. Comparison of the effects of the 5 alpha-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. *Endocrinology* 131:1149–1156.
- Jost A. 1953. Problems of fetal endocrinology: the gonadal and hypophyseal hormones. *Rec Prog Horm Res* 8:379–418.
- Jost A. 1970. Hormonal factors in the sex differentiation of the mammalian foetus. *Philos Trans R Soc Lond B Biol Sci* 259:119–130.
- Kelce WR, Lambright CR, Gray LE Jr, Roberts KP. 1997. Vinclozolin and *p,p'*-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism. *Toxicol Appl Pharmacol* 142:192–200.
- Klein RM. 1986. Models for the study of cell proliferation in the developing gastrointestinal tract. *J Pediatr Gastroenterol Nutr* 5:513–517. [Published erratum *J Pediatr Gastroenterol Nutr* 6:489 (1987)].
- McEwen BS. 1981. Neuronal gonadal steroid actions. *Science* 211:1303–1311.
- Meaney MJ, McEwen BS. 1986. Testosterone implants into the amygdala during the neonatal period masculinize the social play of juvenile female rats. *Brain Res* 398:324–328.
- Meaney MJ, Stewart J. 1981. Neonatal-androgens influence the social play of prepubescent rats. *Horm Behav* 15:197–213.
- Meaney MJ, Stewart J, Poulin P, McEwen BS. 1983. Sexual differentiation of social play in rat pups is mediated by the neonatal androgen-receptor system. *Neuroendocrinology* 37(2):85–90.
- Meisel RL, Ward IL. 1981. Fetal female rats are masculinized by male littermates located caudally in the uterus. *Science* 213:239–242.
- Mylchreest E, Cattley R, Foster PM. 1998. Male reproductive tract malformations in rats following gestational and lactational exposure to DBP: an antiandrogenic mechanism. *Toxicol Sci* 43:47–60.
- Mylchreest E, Sar M, Cattley RC, Foster PM. 1999. Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156:81–95.
- Parks LG, Lambright CS, Orlando EF, Guillette LJ Jr, Ankley GT, Gray LE Jr. 2001. Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicol Sci* 62:257–267.
- Parks L, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow N, et al. 2000. The plasticizer diethylhexyl phthalate induces reproductive malformations by decreasing fetal male rat testosterone synthesis. *Toxicol Sci* 58:339–349.
- Paup DC, Coniglio LP, Clemens LG. 1972. Masculinization of the female golden hamster by neonatal treatment with androgen or 17 β estradiol. *Horm Behav* 3:123–131.
- Pellis SM, Pellis VC. 1997. The prejuvenile onset of play fighting in laboratory rats (*Rattus norvegicus*). *Dev Psychobiol* 31:193–205.
- Phoenix C, Goy R, Gerall A, Young W. 1959. Organizational action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology* 65:369–382.
- Pomerantz SM, Roy MM, Thornton JE, Goy RW. 1985. Expression of adult female patterns of sexual behavior by male, female, and pseudohermaphroditic female rhesus monkeys. *Biol Reprod* 33:878–889.
- Reddy VV, Naftolin F, Ryan KJ. 1974. Conversion of androstenedione to estrone by neural tissues from fetal and neonatal rats. *Endocrinology* 94:117–121.
- Rhees RW, Kirk BA, Sephton S, Lephart ED. 1997. Effects of prenatal testosterone on sexual behavior, reproductive morphology and LH secretion in the female rat. *Dev Neurosci* 19:430–437.
- Richmond G, Sachs BD. 1984. Further evidence for masculinization of female rats by males located caudally *in utero*. *Horm Behav* 18:484–490.
- Schiffer B, Daxenberger A, Meyer K, Meyer HH. 2001. The fate of trenbolone acetate and melengestrol acetate after application as growth promoters in cattle: environmental studies. *Environ Health Perspect* 109:1145–1151.
- Slob AK, Van der Schoot P. 1982. Testosterone induced mounting behavior in adult female rats born in litters of different female to male ratios. *Physiol Behav* 28:1007–1010.
- Stahl F, Gotz F, Dörner G. 1991. The influence of fetal adrenals on the androgen levels during brain differentiation in human subjects and rats. *Exp Clin Endocrinol* 98:131–139.
- Thornton J, Goy RW. 1986. Female-typical sexual behavior of rhesus and defeminization by androgens given prenatally. *Horm Behav* 20:129–147.
- Tobet SA, Baum MJ. 1987. Role for prenatal estrogen in the development of masculine sexual behavior in the male ferret. *Horm Behav* 21:419–429.
- Tobet SA, Dunlap JL, Gerall AA. 1982. Influence of fetal position on neonatal androgen-induced sterility and sexual behavior in female rats. *Horm Behav* 16:251–258.
- U.S. EPA. 2000. Overview of the Vinclozolin Risk Assessment. Available: <http://www.epa.gov/pesticide/reregistration/vinclozolin/> [accessed 11 April 2002].
- Vandenberg JG, Hotchkiss AK. 2001. Interfetal communication and adult phenotype in mice. In: *Chemical Signals in Vertebrates IX* (Marchlewska-Koj A, Lepri JJ, Muller-Schwarze D, eds). New York:Kluwer Academic/Plenum Press, 183–187.
- Vandenberg JG, Huggett CL. 1995. The anogenital distance index, a predictor of the intrauterine position effects on reproduction in female house mice. *Lab Anim Sci* 45:567–573.
- Vandenberg JG, Huggett CL. 1994. Mother's prior intrauterine position affects the sex ratio of her offspring in house mice. *Proc Natl Acad Sci U S A* 91:11055–11059.
- vom Saal FS. 1979. Prenatal exposure to androgen influences morphology and aggressive behavior of male and female mice. *Horm Behav* 12:1–11.
- vom Saal FS, Bronson FH. 1980. Sexual characteristics of adult female mice are correlated with their blood testosterone levels during prenatal development. *Science* 208:597–599.
- vom Saal F, Clark MM, Galey BG, Drickamer LC, Vandenberg JG. 1999. Intrauterine position phenomenon. In: *Encyclopedia of Reproduction* (Knobil E, Neill J, eds). San Diego:Academic Press, 893–900.
- Vreeburg JT, Groeneweld JO, Post PE, Ooms MP. 1983. Concentrations of testosterone and androsterone in peripheral and umbilical venous plasma of fetal rats. *J Reprod Fertil* 68:171–175.
- Waller CL, Juma BW, Gray LE Jr, Kelce WR. 1996. Three-dimensional quantitative structure–activity relationships for androgen receptor ligands. *Toxicol Appl Pharmacol* 137:219–227.
- Weisz J, Ward IL. 1980. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. *Endocrinology* 106:306–316.
- Welshons WV, Nagel SC, Thayer KA, Judy BM, Vom Saal FS. 1999. Low-dose bioactivity of xeno17 β estradiols in animals: fetal exposure to low doses of methoxychlor and other xeno17 β estradiols increases adult prostate size in mice. *Toxicol Ind Health* 15:12–25.
- Wilson JD, Lasnitzki I. 1971. Dihydrotestosterone formation in fetal tissues of the rabbit and rat. *Endocrinology* 89:659–668.
- Wolf CJ, LeBlanc GA, Ostby JS, Gray LE Jr. 2000. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. *Toxicol Sci* 55:15.
- Zielinski WJ, Vandenberg JG. 1991. Effect of intrauterine position and social density on age of first reproduction in wild-type female house mice (*Mus musculus*). *J Comp Psychol* 105:134–139.